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ISOLATION OF A MORPHOGEN THAT INDUCES VESICLE FORMATION IN A CELL LINE DERIVED FROM IMAGINAL WING DISCS OF TRICHOPLUSIA NI

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A factor that promotes vesicle formation (VPF) in an insect cell line TND1 was purified partially from larval hemolymph of the cabbage looper, Trichoplusia ni (Hübner). The estimated molecular mass for VPF was between 20,500 and 37,500 daltons by gel permeation, 22,500 daltons by the Ferguson plot on nondenaturing PAGE and 16,880 daltons on SDS PAGE. VPF fractions were isolated from hemolymph by gel permeation and then subjected to preparative isoelectric focusing. Two active fractions containing the polypeptide from the isoelectric separations had isoelectric points of 6.18 and 6.32 and had specific activities of 34 and 32 vesicles formed per test culture chamber per µg protein, respectively.

The study of imaginal wing discs and their response to hormones in vitro has led to a better understanding of how insect hormones control metamorphosis in holometabolous insects (1, 2). More recently, hormone-responsive cell lines from specific tissues such as wing discs have been established (3). One such cell line, IAL-TND1, was developed from imaginal wing discs of the cabbage looper moth, Trichoplusia ni. The cell line grows in aggregate (clumped) form (Fig. 1A) but the first year in culture grew as multicellular vesicles (Fig. 1B). Addition of larval hemolymph reversed the morphology from multicellular aggregates to multicellular vesicles (4). Preliminary characterization of the hemolymph factor indicated that it was of a polypeptide nature. Moreover, additional information indicated that the transformation of the vesicle to the aggregate form was accompanied by the cells acquiring the capacity to produce 20-hydroxyecdysone (5, 6).

In this chapter, we present information on the isolation and purification of the vesicle promoting factor (VPF) from larval hemolymph. We also show how this cell line, which both produces and responds to 20-hydroxyecdysone, is a favorable model system for studying the mechanism by which a hormone and morphogen interact to control cellular morphogenesis.

I. General Features of the Cell Line and Vesicle Bioassay

The cell line, designated as IAL-TND1, was established from epithelial cells of imaginal wing discs of T. ni. It grows as suspended multicellular vesicles and aggregates. Its kariology is near diploid with a mode of 56 and has a growth rate increase in density of 3- to 8-fold per week (7).

Vesicle promoting activity was assayed by adding the sample (15-60 µg protein) to the cell aggregates in multiwell plates and counting the number of vesicles produced under an inverted microscope. Hemolymph-free medium was used as a control (4). Also, 500 to 600 µg of protein from whole hemolymph was run in the bioassay as a standard.

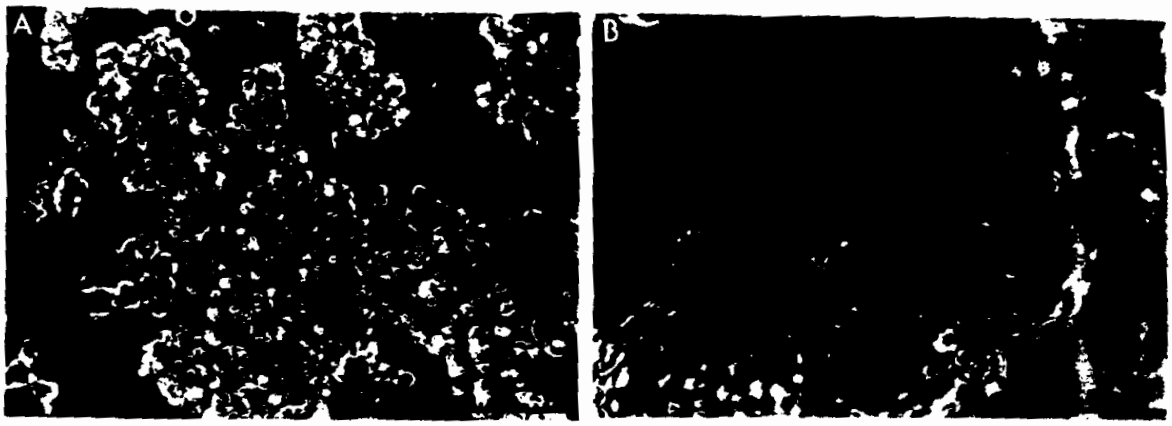

Fig. 1. Results of experiment in which TND1 cells in aggregate form (A) were exposed to 2% T. ni hemolymph that contained VPF and changed to multicellular vesicle form (B) after 6 days of exposure (5).

II. General Features of the Vesicle Promoting Factor

Several properties of the VPF that were determined earlier (4) are as follows: 1) response to hemolymph in the vesicle bioasay is concentration dependent; 2) vesicle promoting activity was present in every developmental stage tested including fourth and fifth instar (last) larvae and pupae; 3) the VPF was detected in three other species of Lepidoptera tested, Spodoptera frugiperda, Galleria mellonella and Manduca sexta, although the greatest activity occurred in T. ni hemolymph; 4) the VPF was tissue specific, with greatest activity in the fat body and the imaginal wing discs; 5) the VPF was heat labile, nondialyzable (10,000 daltons cut off membrane) and sensitive to a nonspecific protease (protease XIV from Streptomyces griseus) but not trypsin. Based on this information, we decided to look for a polypeptide in the larval hemolymph of T. ni. Previously, we reported that exclusion of VPF by dialysis membrane suggested an apparent molecular mass that was greater than 60,000 daltons. However, we know now that VPF readily forms aggregates and that the polypeptide has a molecular mass less than 60,000 daltons (8).

III. Purification of VPF

Initially, hemolymph was separated on a gel permeation column (1000-700,000 daltons fractionation range, Fracto-gel®) (Fig. 2) (8). VPF activity eluted between the 43,000 daltons ovalbumin standard and the 13,500 daltons standard of ribonuclease A. The 25,000 daltons standard of chymotrypsinogen eluted within the area of VPF activity, indicating that the factor had a molecular weight close to this standard. Next, we prepared a calibration curve from a gel with a more narrow fractionation range (500-80,000 daltons). The molecular mass of the VPF was estimated to be between 20,500 to 37,000 daltons. After the gel permeation step, the active fraction was unstable during subsequent separations and storage (8).

The active fractions from the gel permeation column then were lyophilized and separated by preparative isoelectric focusing with a pH range of 5-7. Figure 3A shows the amount of protein in the fractions and Figure 3B shows the bioassay results. Fraction 8 had the most activity, while some activity was present in fraction 9 but not in the other fractions. When we examined these fractions on a nondenaturing polyacrylamide gel, a broad band, labeled b, stained heavier in fraction 8 and lighter in fraction 9. A slower migrating band, labeled a, also was present in fractions 8 and 9, but did not have vesicle promoting activity. Fraction 7 (Fig. 3B), which was applied to lane 1 (Fig. 3C) contained band a but not b, and did not have VPF activity. When fractions 8 and 9 were run on an SDS

dissociating gel, the most evident bands were a 108,000 dalton band and a 73,000 dalton band and a broad, heavy staining band with a molecular mass of 16,880 daltons. Because the gel permeation data indicated the VPF was a low molecular weight polypeptide, we suspected that the 16,880 dalton band was the active band and that it originated from band b on the nondenaturing gel in Figure 3C. When band a was cut from the nondenaturing gel and run on an SDS gel (Fig. 4), only a 73,000 dalton band was evident (lanes 2, 4 & 6). When band b was cut from the nondenaturing gel and run on an SDS gel, only a 16,880 dalton band was evident. As indicated in Figure 3B,C, fraction 7, containing band a, did not have VPF activity but fractions 8 and 9, containing band b, (16,880 daltons) had vesicle promoting activity. A band with an Rf similar to band b also was purified by chromatofocusing and shown to have VPF activity (8).

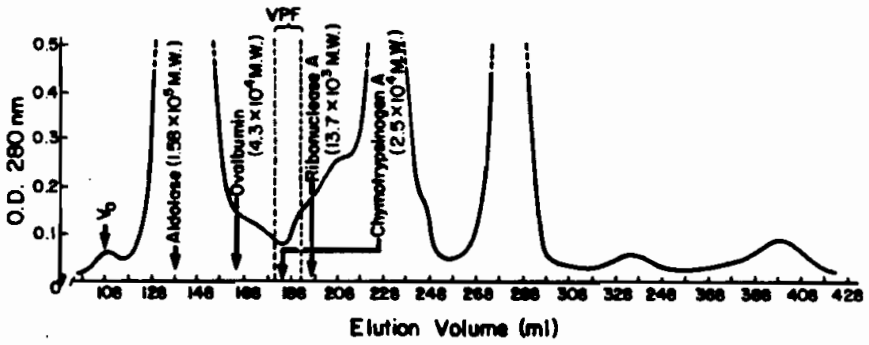

Fig. 2. Molecular-sieve chromatography of cabbage looper larval hemolymph on Fracto-gel. VPF indicates tubes that contain vesicle promoting activity in cell bioassay (8).

The molecular weight of the active fraction 8 from the isoelectric focusing separation (Fig. 3B) was estimated on nondenaturing polyacrylamide gels using the Ferguson plot. The apparent molecular weight of the broad band observed in Figure 3C (band b) had a molecular weight of 22,500 daltons (8).

In summary, the estimated molecular weight for VPF was between 20,500 and 37,500 daltons by gel permeation, 22,500 daltons by the Ferguson plot on nondenaturing PAGE and 16,880 daltons on SDS PAGE (8).

Of the total hemolymph protein, the VPF comprises 0.75% and was active in the VPF bioassay at 10^{-6} M.

V. Inhibitors

As an initial approach to the mode of action of VPF we tested various inhibitors of cellular processes for their effect on vesicle formation (Table 1). Colchicine and vinblastin, which inhibit microtubule formation and prevent ecdysone-induced tracheole migration in imaginal wing discs (9) also inhibited vesicle formation. On the other hand, cycloheximide, a protein synthesis inhibitor, cytochalasin B, a contractile microfilament inhibitor, and tunicamycin, an inhibitor of glycosylation, did not affect vesicle formation. Actinomycin D was toxic to the cells. Additional information is needed at the ultrastructural level of the cells to interpret the effects of the inhibitors on vesicle formation. However, the inhibitory effects of colchicine and vinblastin suggested that the formation of vesicles is dependent on the integrity of the microtubules.

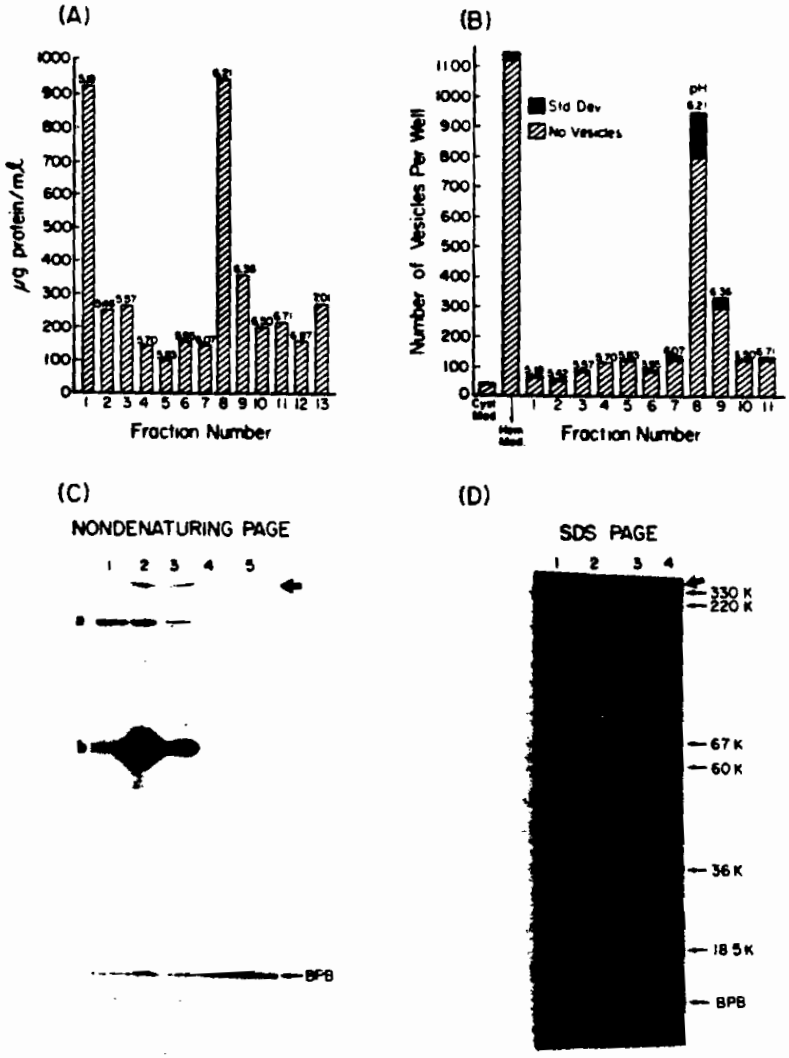

Fig. 3. Isoelectric focusing of combined fractions that contain VPF from molecular-sieve chromatography of cabbage looper larval hemolymph. (A) Protein content and pH of fractions. (B) VPF activity and pH of fractions. (C) Nondenaturing PAGE of fractions 7, 8, 9, 10 and 11 in lanes 1, 2, 3, 4, and 5, respectively. BPB, bromophenol blue marker. (D) SDS PAGE of fractions 8 and 9 in lanes 2 and 3. Molecular weight standards in lanes 1 and 2: thyroglobulin (330 Kd), ferritin (220 Kd), albumin (67 Kd), catalase (60 Kd), lactate dehydrogenase (36 Kd), ferritin subunit (18.5 Kd) and BPB, bromophenol blue marker. Heavy arrows in C and D indicate surface of separating gel. Gels (12.5 %) were stained with Coomassie blue (8).

Table 1. Effects of inhibitors on hemolymph-induced vesicle formation.

Inhibitor	Concentration (µg/ml)	% of Hemolymph control
Colchicine	0.02	33.3
	0.2	12.0
Vinblastine	0.02	13.4
	0.2	8.5
Cycloheximide	0.2	100.0
	2.0	100.0
Cytocholasin B	2.0	100.0
	20.0	100.0
Tunicamycin	0.02	100.0
	0.2	100.0
Actinomycin D*	0.02	0.0
	0.2	0.0

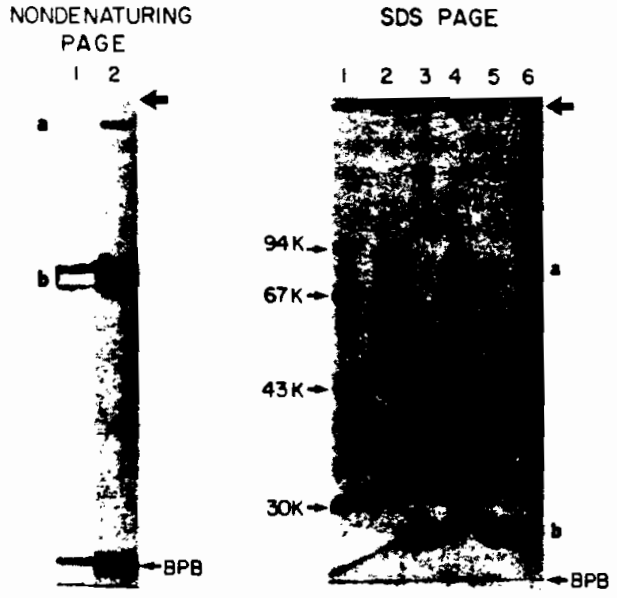

Fig. 4. Protein bands from a VPF active fraction cut out of nondenaturing polyacrylamide gel and run on SDS PAGE. The VPF fraction was isolated by preparative isoelectric focusing as described in Figure 2. Nondenaturing PAGE: lanes 1 and 2, VPF active fraction from isoelectric focusing separation, isoelectric point 6.21. The bands in lane 1 were cut out of the gel and placed on the surface of the SDS gel and then electrophoresed. SDS PAGE: lane 1, molecular weight standards, carbonic anhydrase (30 Kd); lanes 2, 4 and 6 band labeled a from lane 1, nondenaturing PAGE; and lanes 3 and 5 band labeled b from lane 1, nondenaturing PAGE. BPB, bromophenol blue marker, stained with Coomassie blue.

V. Ecdysteroids

20-Hydroxyecdysone at physiological levels of the hormone (0.1 and 1.0 µg/ml) was inhibitory to vesicle formation (6). 22-Iso-ecdysone, which is a known inactive analog of the hormone in the molting process, also was inactive in the assay. Thus, the hormone specificity and concentration of 20-hydroxyecdysone required for inhibition of the response are similar to that of a positive response to the hormone in wing discs in culture.

Additionally, the aggregate form of the cell line is now known to produce 20-hydroxyecdysone (5, 6). It was found that imaginal wing discs which were cultured in aggregate-conditioned medium changed morphologically in a manner similar to treatment with physiological levels of 20-hydroxyecdysone by undergoing evagination and peripodial membrane separation, later steps in disc development (5). Since the VPF induces the aggregate form to produce vesicles and the effect of VPF can be overridden by addition of physiological levels of 20-hydroxyecdysone, the question then becomes, "does VPF destroy the ecdysteroids produced by the cells?" This hypothesis was tested by incubating hemolymph with 20-hydroxyecdysone and then placing imaginal discs in the medium to see if they would exhibit some of the characteristic responses to ecdysteroids (5). Two percent hemolymph plus 500 ng or 10^{-6} M 20-hydroxyecdysone did not prevent action of the hormone. When the hemolymph concentration was raised to 20% and the conc. of hormone lowered 10-fold, the hormone was still effective. Therefore, VPF did not appear to act by destroying or inactivating the hormone, and must induce vesicle formation by some other means.

VI. Conclusions

The cell line and the VPF provide a promising model system for studying the control of cell morphogenesis in vitro. We have isolated and purified a polypeptide that induces vesicle formation and an inhibition of this process can be effected by addition of 20-hydroxyecdysone. The relationship of this in vitro system to development in vivo is the subject of continued investigation. Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by the USDA.

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